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Impaired expression of key molecules of ammoniogenesis underlies renal acidosis in a rat model of chronic kidney disease

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Abstract: BACKGROUND: Advanced chronic kidney disease (CKD) is associated with the development of renal metabolic acidosis. Metabolic acidosis per se may represent a trigger for progression of CKD. Renal acidosis of CKD is characterized by low urinary ammonium excretion with preserved urinary acidification indicating a defect in renal ammoniogenesis, ammonia excretion or both. The underlying molecular mechanisms, however, have not been addressed to date. METHODS: We examined the Han:SPRD rat model and used a combination of metabolic studies, mRNA and protein analysis of renal molecules involved in acid-base handling. RESULTS: We demonstrate that rats with reduced kidney function as evident from lower creatinine clearance, lower haematocrit, higher plasma blood urea nitrogen, creatinine, phosphate and potassium had metabolic acidosis that could be aggravated by HCl acid loading. Urinary ammonium excretion was highly reduced whereas urinary pH was more acidic in CKD compared with control animals. The abundance of key enzymes and transporters of proximal tubular ammoniogenesis (phosphate-dependent glutaminase, PEPCK and SNAT3) and bicarbonate transport (NBCe1) was reduced in CKD compared with control animals. In the collecting duct, normal expression of the B1 H(+)-ATPase subunit is in agreement with low urinary pH. In contrast, the RhCG ammonia transporter, critical for the final secretion of ammonia into urine was strongly down-regulated in CKD animals. CONCLUSION: In the Han:SPRD rat model for CKD, key molecules required for renal ammoniogenesis and ammonia excretion are highly down-regulated providing a possible molecular explanation for the development and maintenance of renal acidosis in CKD patients.

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Impaired expression of key molecules of ammoniogenesis underlies renal acidosis in a rat model of chronic kidney disease

Running title: Impaired ammonium excretion in CKD

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ABSTRACT

Background: Advanced chronic kidney disease (CKD) is associated with the development of renal metabolic acidosis. Metabolic acidosis per se may represent a trigger for progression of CKD. Renal acidosis of CKD is characterized by low urinary ammonium excretion with preserved urinary acidification indicating a defect in renal ammoniogenesis, ammonia excretion or both. The underlying molecular mechanisms, however, have not been addressed to date.

Methods: We examined the Han:SPRD rat model and used a combination of metabolic studies, mRNA and protein analysis of renal molecules involved in acid-base handling.

Results: We demonstrate that rats with reduced kidney function as evident from lower creatinine clearance, lower hematocrit, higher plasma BUN, creatinine, phosphate and potassium, had metabolic acidosis that could be aggravated by HCl acid loading. Urinary ammonium excretion was highly reduced whereas urinary pH was more acidic in CKD compared to control animals. The abundance of key enzymes and transporters of proximal tubular ammoniogenesis (PDG, PEPCK, SNAT3) and bicarbonate transport (NBCe1) was reduced in CKD compared to control animals. In the collecting duct, normal expression of the B1 H⁺-ATPase subunit is in agreement with low urinary pH. In contrast, the RhCG ammonia transporter, critical for the final secretion of ammonia into urine was strongly downregulated in CKD animals.

Conclusion: In the Han.SPRD rat model for CKD key molecules required for renal ammoniagenesis and ammonia excretion are highly downregulated providing a possible molecular explanation for the development and maintenance of renal acidosis in CKD patients.

INTRODUCTION

Metabolic acidosis is frequent in chronic kidney disease (CKD) and is associated with several complications such as muscle wasting, impaired growth in children, bone disease, hypo-albuminemia, inflammation, and insulin resistance. Moreover it has been associated with increased mortality in dialysis and non-dialysis dependent CKD patients [1-5]. Numerous recent studies particularly highlighted the role of metabolic acidosis in the progression of CKD and the increased risk to develop end stage renal disease [6-9]. The mechanisms of metabolic acidosis in CKD have been first functionally investigated more than 50 years ago and it was shown that patients with metabolic acidosis and CKD demonstrate reduced excretion of ammonium, the most important buffer for renal acid excretion and bicarbonate synthesis, with preserved ability to acidify urine [10-13]. However, the affected molecular pathways in the development of metabolic acidosis in CKD have not been addressed to date.

Hannover rat (Han:SPRD) is a commonly used model for autosomal dominant polycystic kidney disease although the disease is caused by a mutation in the *Pkdr1* gene and no mutations are found in humans with polycystic kidney disease (3, 4). Heterozygous male (cy/+) animals are characterized by a slow progression of kidney disease and proteinuria [14, 15]. Consequently, these rats have also been used as a model for chronic kidney disease [16, 17].

Renal ammoniagenesis and ammonium excretion depend on a complex interplay between different nephron segments [18-21]. Ammoniogenesis in the proximal tubule serves mostly the de novo synthesis of bicarbonate to replenish

bicarbonate buffers used by metabolism. Glutamine is taken up by proximal tubule cells, most likely by the SNAT3 amino acid transporter and metabolized by a series of enzymes located in mitochondria and cytosol. Phosphate-dependent glutaminase (PDG) and glutamate dehydrogenase (GDH) release two ammonia (NH_3) molecules and one bicarbonate (HCO_3^-) ion whereas further metabolism of the resulting alpha-ketoglutarate by the phospho-enol pyruvate carboxy kinase (PEPCK) generates an additional HCO_3^- ion. The process of proximal tubular ammoniagenesis is highly stimulated during acidosis or by acid-load [21, 22]. Ammonium (NH_4^+) produced by the proximal tubule is mostly released into urine and is partly reabsorbed at the level of the thick ascending limb of the loop of Henle, concentrated in the medullary interstitium and secreted into urine [18-20]. The final secretory step depends on the capacity of H^+ -ATPases to acidify urine and the RhCG ammonia transporter [18, 19, 23].

Here, we used the Han:SPRD rats as a CKD model and investigated the regulation of key molecules in renal acid-base handling to identify the molecular mechanisms that contribute to the development of metabolic acidosis in chronic kidney disease. Our data demonstrate a strongly reduced expression of enzymes and transporters involved in proximal ammoniagenesis and ammonia secretion whereas the capacity to acidify urine was preserved. Thus, our data suggest that metabolic acidosis is caused by the kidneys' impaired ability to produce and excrete adequate ammonium amounts thereby impairing bicarbonate synthesis and metabolic acid buffering.

MATERIALS AND METHODS

Animal Model

The study was conducted in 4 months old male heterozygous (cy/+) and wild type (+/+) littermate Han:SPRD rats. Rats received standard diet (Provimi Kliba S.A., Switzerland) at day 1 and 2, and consecutively HCl diet (500 g standard diet mixed with 750 ml of 333 mM HCl) to induce an additional acid-load or wet standard diet (analogously mixed with distilled water) for 2 days (days 3 and 4). Rats were placed into metabolic cages on day 1 for adaptation. Water and food intake as well as body weight were daily measured, and 24h-urine (under mineral oil) and stool were collected. All animal experiments were performed according to national and international guidelines and laws of animal welfare and protocols approved by the local Veterinary Authority (Veterinäramt Zürich). At the end of the experiments rats were anesthetized with isoflurane, heparinized blood was collected from the tail artery, and animals were perfused with PBS (136.9 mM NaCl, 2.68 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4 with HCl) to remove blood. Kidneys were immediately removed and subsequently snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

Blood and Urine Analysis

Rats were anesthetized with isoflurane (IsoFlo[®], Abbott AG, Baar, Switzerland), 1.5 – 2.0 %, and heparinized blood was collected from the tail artery and analyzed immediately for pH, blood gases, and electrolytes on a

Radiometer ABL 800 Flex blood gas analyzer (Radiometer, Copenhagen, Denmark) on day 2, 3 and 5. In addition, at the end of the experiment (day 5) blood was taken from the left ventricle and the inferior caval vein to measure plasma creatinine, phosphate, BUN, glucose, and albumin using the clinical chemistry analyzer Piccolo[®] Xpress (Abaxis, Union City, CA). Ion chromatography (Metrohm ion chromatograph, Herisau, Switzerland) was performed to determine urinary Na⁺, K⁺, and Cl⁻ concentrations. Urine pH, pCO₂, and calculated HCO₃⁻ were determined by aspirating urine from the collectors into gas-tight syringes and injecting it into the blood gas analyzer. Urinary creatinine was analyzed using the Jaffé method [24, 25]. Urinary ammonium concentration was determined using the Berthelot protocol [26]. Urinary phosphate was measured by standards of Urine Precision Controls (Randox Lab., UK). Creatinine clearance was calculated from the measured values.

RNA extraction

Approximately 25 mg of snap-frozen kidney tissue was homogenized in RLT-Buffer (Qiagen, Hilden, Germany) supplemented with 2-mercaptoethanol to a final concentration of 1 %. Total RNA was extracted from 600 µl of each homogenized sample using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. DNase digestion was performed using the RNase-Free DNase Set (Qiagen). Quality, purity, and concentration of the isolated RNA preparations were analyzed spectroscopically using the ND-1000

spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA samples were stored at – 80 °C.

Semi-quantitative real-time RT-PCR

The RNA samples were diluted to 100 ng/μl. First, reverse transcription was performed using the TaqMan Reverse Transcription Reagents (Roche Molecular Systems, Branchburg, NJ/Applied Biosystems). Briefly, 12.4 μl RNase-free water, 4 μl 10X RT Buffer, 8.8 μl MgCl₂ Solution, 2 μl Random Hexamers, 8 μl dNTP Mixture, 0.8 μl RNase Inhibitor, and 1 μl MultiScribe Reverse Transcriptase (respectively 1 μl of additional RNase-free water for negative controls) were mixed with 3 μl of RNA to perform reverse transcription by using the *Tpersonal* Combi thermocycler (Biometra, Göttingen, Germany) with thermocycling conditions set at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. Next, relative mRNA expression was determined using quantitative real-time PCR (qRT-PCR) with the 7500 Fast Real-Time PCR System (Applied Biosystems) on the basis of the standard 7500 run mode. Primers for all genes of interest were designed using Primer3-web software (frodo.wi.mit.edu/primer3) (see supplementary table 1). Probes of the tested genes were labeled with the reporter dye FAM at the 5' end and the quencher dye TAMRA at the 3' end (Microsynth, Balgach, Switzerland). The probe of the housekeeping gene Human 18S rRNA (Applied Biosystems, Foster City, CA) was labeled with the reporter dye VIC at the 5' end and the quencher dye TAMRA at the 3' end. The specificity of all primers was first tested in a standard PCR and always resulted in a single

product of the expected size on 1.5 % agarose gels (data not shown). Briefly, 2 µl cDNA, 0.8 µl of each primer (25 µM), 0.4 µl labeled probe (5 µM), 6 µl RNase-free water, and 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems) reached 20 µl of final reaction volume for the tested gene. The protocol for the housekeeping gene was slightly different as the following: 2 µl cDNA, 1 µl of the Human 18S rRNA (20X) Control primers and probe reagent, 7 µl RNase-free water, and 10 µl TaqMan Universal PCR Master Mix. Reaction conditions were: Denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation at 60 °C for 60 s with autoramp time. All reactions were run in triplicates, including a negative control. To analyze the data, we set the threshold to 0.06 as this value had been determined to be in the linear range of the amplification curves for all mRNAs in all experimental runs. The expression of gene of interest was calculated in relation to Human 18S rRNA. Relative expression ratios were calculated as $R = 2^{[Ct(18S) - Ct(\text{gene of interest})]}$, where Ct represents the cycle number at the threshold 0.06.

Western Immunoblotting

For immunoblotting brush border membrane (BBM) proteins were prepared using the Mg^{2+} precipitation technique [27]. Additionally, protein extraction of total membrane fractions (MF) of kidney tissue was performed. Briefly, kidney tissue was homogenized in ice-cold HEPES buffer (200 mM mannitol, 80 mM HEPES, 41 mM KOH, titrated with KOH to pH 7.5) with Protease Inhibitors Cocktail Tablets complete, Mini (Roche Diagnostics GmbH,

Mannheim, Germany). Both BBM and MF protein samples were used for Western Blotting. After measurement of the protein concentration (Bio-Rad Dc Protein Assay, Bio-Rad, Hercules, CA), 40 µg of MF protein or 20 µg of BBM protein was solubilized in Laemmli sample buffer, and SDS-PAGE was performed on 8 – 10 % polyacrylamide gels. Proteins were transferred electrophoretically from gels to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). After blocking with 5 % milk powder in Tris-buffered saline containing 0.1 % (v/v) Tween-20 for 60 min at room temperature, the blots were incubated with the respective primary antibodies overnight at 4 °C or 2 h at room temperature (see supplementary table 2). Antibodies against SNAT produced two bands, a specific band of approximately 55-60 kDa and an unspecific band of lower molecular weight of approximately 40 kDa. PDG appears as two bands of 66 and 68 kDa and both bands were quantified together [28]. Similarly, pendrin antibodies recognized two specific bands of approximately 100 and 150 kDa [29].

After washing and subsequent blocking, blots were incubated with the secondary antibody [donkey anti-rabbit or sheep anti-mouse antibodies linked to horseradish peroxidase 1:10,000 (GE Healthcare, Little Chalfont, Buckinghamshire, UK) or goat anti-rabbit and goat anti-mouse antibody 1:5,000 linked to alkaline phosphatase (Promega, Madison, WI) for 1 h at room temperature. Antibody binding was detected with appropriate Immobilon Western chemiluminescence substrates (Millipore, Billerica, MA), using the LAS-4000 Luminescent Image

Analyzer (Fujifilm, Tokyo, Japan). All images were analyzed using appropriate software (Advanced Image Data Analyzer version 3.44, Raytest) to calculate the protein of interest/actin ratio.

Statistical Analysis

All data are presented as means \pm SE. Unpaired two-tailed *t*-test or ANOVA with Bonferroni correction were applied for inference statistics and result with *p-value* < 0.05 were considered statistically significant.

RESULTS

Han:SPRD cy/+ rats recapitulate hallmarks of chronic kidney disease and develop severe hyperchloremic metabolic acidosis

Cy/+ compared to +/+ rats receiving standard diet had lower blood bicarbonate, higher chloride, and reduced $p\text{CO}_2$ levels indicating a partly compensated hyperchloremic metabolic acidosis. Potassium and phosphate plasma levels were elevated and creatinine clearance and hematocrit were lower demonstrating advanced chronic kidney disease (tables 1 and 2) in cy/+ rats. Urine analysis showed more acidic urine, lower urinary bicarbonate, and higher phosphate excretion in cy/+ compared to +/+ rats whereas ammonium excretion was similar between groups (table 2). Thus, cy/+ rats display the major hallmarks of advanced chronic kidney disease confirming and expanding previous reports [30, 31].

Next we tested the rats ability to respond to an additional acid load administrated by adding HCl to food for 48 hrs. Arterial blood gas analysis confirmed induction of metabolic acidosis in HCl-treated animals (table 1). However, cy/+ rats developed a more severe metabolic acidosis compared to +/+ littermates (blood pH 7.13 ± 0.00 vs. 7.34 ± 0.01). Accordingly, arterial bicarbonate levels were lower in cy/+ animals compared to +/+ rats (8.3 ± 0.4 vs. 15.6 ± 0.3 mmol/l). These results indicate, that cy/+ animals' ability to buffer an

acid load is impaired. Interestingly, induction of acidosis was associated with a fall in creatinine clearance in both wildtype and cy/+ animals (table 2).

The dietary acid load caused a markedly acidic urinary pH in both cy/+ and +/+ rats (table 2). Thus, also cy/+ rats with markedly reduced renal function were able to acidify urine to the same extent as wildtype animals with normal renal function. Similar, patients with advanced CKD maintain their ability to acidify urine [10, 12] suggesting that the Han:SPRD rat model serves as an useful CKD model for studying metabolic acidosis.

After acid load urinary ammonium excretion, a major component of overall net acid excretion, was significantly lower in cy/+ compared to +/+ rats (table 2) whereas, urinary phosphate excretion, a major component of titratable acidity, increased modestly in both animal groups. Our data suggest that urinary acidification is preserved in CKD animals whereas urinary ammonium excretion is decreased by approximately 60 – 80 % which is paralleled by more severe metabolic acidosis.

Expression of ammoniagenic key molecules is reduced in CKD rats

We performed real-time RT-qPCR on kidney tissue for SNAT3, PDG, PEPCK, and the sodium/proton exchanger type 3 (NHE3), key molecules of the renal ammoniogenesis pathway. The mRNA abundance of PEPCK, SNAT3, and NHE3 was reduced in cy/+ CKD rats (figure 1) compared to wildtype animals under standard diet and after 48 hrs acid load. In contrast, PDG mRNA expression was increased after the acid load in cy/+CKD animals (figure 1).

Similarly protein abundance of SNAT3 and PEPCK in cy/+ animals was markedly decreased compared to wildtype littermates (figure 2). PDG protein levels were decreased after the acid load, opposite to mRNA levels (figure 2). Protein levels of NHE3 were not different compared to wildtype animals (figure 2).

The electrogenic sodium/bicarbonate cotransporter type 1 (NBCe1) is located on the basolateral membrane of the proximal tubule cell and releases bicarbonate into blood. mRNA expression of NBCe1 was decreased in cy/+ CKD animals under standard diet and increased after acid load (figure 3). Protein levels were also decreased under both standard diet and after the acid load (figure 3).

Taken together, these data demonstrate a strong reduction in the expression of key molecules of the ammoniogenesis pathway in kidneys from cy/+ CKD animals under standard diet. CKD animals were able to modestly increase urinary ammonium excretion after acid loading, however, to a much lesser extent than wildtype animals. Thus, reduced expression of key molecules of the ammoniogenesis pathway parallels lower urinary ammonium excretion in CKD rats.

Sodium-dependent phosphate transporters are altered in CKD rats

The major renal phosphate transporters NaPi-IIa and NaPi-IIc and the third sodium-dependent phosphate transporter Pit-2 are expressed on the apical membrane of the proximal tubule cells and mediate phosphate reabsorption [32]. mRNA abundance of NaPi-IIa and NaPi-IIc was decreased during standard diet

and after acid loading in cy/+ rats compared to control rats (figure 4) while Pit-2 mRNA expression was not altered. Brush border membrane protein levels of the three phosphate transporters were differently regulated. NaPi-IIc protein abundance was decreased while NaPi-IIa and Pit.2 remained unchanged. However, after an acid load Pit-2 protein levels were increased (figure 5).

Ammonium excretion is reduced in CKD rats

The last set of experiments examined the abundance of proteins contributing to final urinary acidification and ammonium excretion along the collecting duct system: the type A intercalated cell specific AE1 anion exchanger, the non-type A intercalated cell marker pendrin, the ammonia transporter RhCG, and the B1 subunit of the H⁺-ATPase enriched in intercalated cells [23]. The mRNA abundance of AE1 was decreased in CKD animals while B1 mRNA abundance was markedly increased after acid loading compared to wildtype animals (figure 6). RhCG and pendrin mRNA abundance was not different. AE1 protein levels were reduced in CKD rats while B1 protein levels were unchanged in comparison to wildtype rats (figure 7). RhCG protein abundance was strongly decreased under standard diet and after acid loading compared to wildtype animals. Thus, normal H⁺-ATPase expression and reduced RhCG abundance are consistent with the preserved urinary acidification and reduced ammonium excretion in CKD rats.

DISCUSSION

Metabolic acidosis is a common complication of CKD and is associated with increased mortality in dialysis and non-dialysis dependent CKD patients. Particularly, the impact of metabolic acidosis on the progression of CKD and the increased risk to develop end stage renal disease have been recently highlighted by studies investigating the effect of alkali supplementation as a potential nephroprotective treatment in patients with CKD (5,11,14).

The major findings in our study are: i) *cy/+* Han:SPRD rats mimic major features of CKD, ii) metabolic acidosis in this CKD rat model is paralleled by the reduced expression of key molecules of proximal tubular ammoniogenesis, iii) urinary acidification and expression of H⁺-ATPases in the collecting duct are preserved, iv) the RhCG ammonia transporter required for final urinary excretion of ammonium is downregulated, and v) renal phosphate reabsorption was reduced in CKD rats.

Han:SPRD rats mimic features of chronic kidney disease

Humans with CKD have reduced GFR and develop several complications such as anemia, metabolic acidosis, mineral bone disease, neuropathy etc. with progressive disease [33]. Similarly, *cy/+* rats presented with chronic kidney disease confirmed by elevated creatinine and BUN values in blood and reduced creatinine clearance. *Cy/+* rats had also metabolic acidosis, lower hematocrit, hyperkalemia and hyperphosphatemia. These findings are consistent with and

expand previous reports using this rat strain as model for CKD or polycystic kidney disease [30, 31]. Acid loading wildtype and cy/+ rats, caused a fall in creatinine clearance in both groups. The mechanism(s) by which acidosis may reduce GFR is currently not exactly known.

Metabolic acidosis in chronic kidney disease is due to reduced expression of key molecules of ammoniagenesis and decreased ammonium excretion by the kidney

Patients with CKD and metabolic acidosis maintain their ability to excrete acidic urine but have a reduced capacity to increase their ammonium excretion [11, 12]. Ammoniogenesis is markedly reduced in patients with CKD which correlates with decreased renal metabolism of glutamine [34]. However, whether reduced total ammoniogenesis reflects only a lower number of nephrons or a decreased capacity of surviving nephrons has been intensively studied in rat models of partial nephrectomy or infarction [13, 35]. These experiments indicated that the kidneys capacity to produce ammonium was proportional to nephron number, ammoniogenesis could be stimulated by diet and that a compensatory increase in ammonium producing cells could be found. In our experiments urinary ammonium excretion was inappropriately low in CKD rats and responded only with a small increase to the acid load. This may result from impaired ammoniogenesis, defective ammonium excretion or urinary acidification, or a combination of these factors. mRNA abundance and protein expression of PEPCK and SNAT3 were highly reduced in CKD rats. Additionally, PDG and

NBCe1 protein abundance were also decreased in acid-loaded CKD rats. Of note, the direction of changes in mRNA expression and protein abundance differed for NHE3, PDG, NBCe1, NaPi-IIa, and Pit-2 for some conditions. The causes for these differences are currently not known but these differences indicate that the interpretation of changes in mRNA expression without analysis of protein abundance can be misleading.

SNAT3 is expressed on the basolateral membrane of proximal tubule cells mediating glutamine uptake for ammoniagenesis [36-38]. Glutamine is further metabolized by a series of enzymes including phosphate dependent glutamine (PDG), glutamate dehydrogenase (GDH), and finally phosphoenol pyruvate carboxy kinase (PEPCK) resulting in the cumulative synthesis of two NH_3 and two HCO_3^- ions [21]. Bicarbonate generated during these metabolic steps is released into blood by the basolaterally located NBCe1 sodium-bicarbonate cotransporter. Reduced expression of all these key molecules in the cy/+CKD rats may severely impair ammoniagenesis and bicarbonate transport.

We and others have previously shown that many of these transporters and enzymes investigated here respond with changes in their expression to endogenous or exogenous acid loads. The expression of NHE3, NaPi-IIa, PDG, PEPCK, SNAT3, NBCe1, RhCG, and AE1 is increased whereas pendrin expression decreased in kidneys from acid-loaded kidneys [39-42]. Here, we compared only control animals vs cy/+CKD rats as we wanted to assess how renal impairment impacted on the ability of the kidney to excrete acid but were not interested to study the renal response per se to an acid load. However, the

regulation can be inferred from the ratios between WT and Cy/+ CKD animals at baseline and after the acid load showing that most proteins were upregulated in the Cy/+ CKD rats with the acid load as the ratios remain constant, e.g. for SNAT3, NHE3, NBCe1, NaPi-IIa, NaPi-IIc, AE1, B1, and RhCG. In the case of pendrin, acid-loading reduced pendrin expression in WT to the same level as in Cy/+ under baseline conditions most likely reflecting the already existing acidosis in CKD rats. In contrast, acid-loading revealed a strong increase in Pit-2 expression in CKD rats and reversed a trend to higher expression of PDG in CKD rats to a lower expression in acid-loaded CKD rats.

We assessed the abundance of these molecules in tissue homogenates from whole kidneys. Thus, our results do not directly indicate whether reduced abundance is due to reduced nephron number or rather due to decreased expression per nephron. Data from animal models with reduced kidney mass suggested that the reduced number of functional nephrons would decrease the total capacity to produce ammonium and that the rate of ammoniagenesis was even stimulated at the level of the single nephron [13, 35]. However, our results may be also interpreted as a downregulation of key molecules of ammoniagenesis. Reduced expression was found only for few molecules such as SNAT3, PEPCK, NBCe1 and NaPi-IIc whereas other proximal tubule proteins such as NaPi-IIa, Pit2, NHE3, or PDG remained unaltered or were even up-regulated. Therefore, the results may suggest that reduced expression of SNAT3, PEPCK, and NBCe1 may be caused by a downregulation in surviving nephrons. In fact, the degree of metabolic acidosis found in CKD rats at baseline would

cause a strong upregulation of these enzymes and transporters in the healthy kidney [22]. The mechanisms that may induce a specific downregulation of some proximal tubular proteins involved in ammoniogenesis and bicarbonate transport remain to be identified. Clearly, this important question warrants further experiments in this CKD animal model as well as in additional models to clarify the underlying mechanisms and whether these mechanisms are model specific or occur in different models of CKD and also in humans.

Accumulation of ammonium in the medullary interstitium is necessary to facilitate excretion into the collecting duct. The transport of ammonium into the interstitium is mediated by the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -cotransporter NKCC2 and the Na^+/H^+ exchanger NHE4 and may involve interactions with sulfatides [18, 43, 44]. We tested the expression of NKCC2 and found no differences between control and Cy/+ CKD rats (data not shown). However, we cannot rule out that expression of NHE4 (for which no specific antibodies exist) or the interstitial concentration of ammonium were not altered.

Ammonium excretion and final urinary acidification are mediated by the late distal convoluted tubule, connecting tubule, and collecting duct [23]. Type A intercalated cells secrete protons and ammonia whereas neighboring segment-specific cells (principal cells) also contribute to ammonia secretion [19, 23, 45]. The final step of ammonia excretion depends on the generation of a corticopapillary gradient of ammonia from interstitium into the lumen of the collecting duct [20, 43]. Ammonia secretion is mediated by the two rhesus proteins RhBG

and RhCG where RhCG appears to play a major role [45, 46]. The driving force for ammonia secretion, however, depends on the degree of urinary acidification by type A intercalated cells. The importance of acid-base transport proteins in these cells is highlighted by inherited disorders in humans with mutations in the AE1/SLC4A1 chloride/bicarbonate exchanger, and the B1/ATP6V1B1 and a4/ATP6V0A4 H⁺-ATPase subunits [47-50]. Urinary pH was more acidic in cy/+ CKD rats under baseline conditions and was further acidified after the HCl diet demonstrating an intact capacity to generate and maintain a steep proton gradient from interstitium to the collecting duct lumen. Surprisingly, AE1 mRNA and protein abundance was decreased in CKD animals whereas the abundance of the B1 H⁺-ATPase subunit remained unaltered. Thus, the maximal capacity of the collecting duct to maximally acidify urine may be decreased, however, in the presence of greatly reduced ammonia concentrations urinary pH remains very acidic. The reduced expression of pendrin, a marker of non-type A intercalated cells is most likely caused by metabolic acidosis [29, 51, 52]. Impaired ammonia secretion by the collecting duct may not only be the result of decreased proximal tubular ammoniagenesis but also due to lower expression of the ammonia transporter RhCG. Thus, reduced secretion of ammonia by the collecting duct is likely by a combination of decreased ammoniagenesis and reduced RhCG expression. The reason for lower RhCG expression is not known and may be either the direct consequence of renal disease or a response to reduced ammoniagenesis. Similarly, in a mouse model with haploinsufficiency of Rhcg,

reduced urinary acid excretion was observed indicating that low levels of RhCG expression per se may reduce renal acid excretion [45].

Renal reabsorption of phosphate is reduced in HAN:SPRD rats

Efficient proton secretion depends in part on the availability of titratable acids, mostly phosphate, that buffer protons and thereby prevent an excessive urinary acidification that would impair further acid excretion (23). In metabolic acidosis phosphate excretion is increased to increase buffer capacity and to reduce extracellular phosphate derived from bone [53]. Phosphate excretion in the kidney is mainly controlled by reabsorption of phosphate in the proximal tubule mediated by a set of sodium dependent transporter, namely NaPi-IIa and NaPi-IIc [32]. Protein abundance of NaPi-IIc but not of NaPi-IIa was down-regulated in CKD rats after HCl load. Urinary phosphate excretion was higher after acid loading. Moreover, CKD rats had increased phosphaturia under normal conditions that was paralleled by reduced expression of NaPi-IIc but not NaPi-IIa. Thus, phosphaturia under these conditions may result from a combination of direct inhibition of NaPi-IIa and NaPi-IIc transport activity by lower urinary pH in the proximal tubule due to lower plasma bicarbonate levels and the downregulation of NaPi-IIc [39]. The upregulation of Pit-2 abundance in acid-loaded cy/+ rats may reflect a compensatory mechanism to limit urinary phosphate losses.

In conclusion, we demonstrated that renal ammonium excretion is severely impaired in a CKD rat model while urinary acidification is mostly preserved. The expression of key molecules of the ammoniogenesis pathway in the proximal tubule and the excretory ammonium transporter RhCG is reduced in kidney. Whether the reduced number of functional nephrons and/or the downregulation of these molecules cause the renal acidosis in CKD remains to be further explored.

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Conflict of interest

The authors declare that they are not aware of any conflicts of interest and that the results presented in this paper have not been published previously in whole or part, except in abstract format.

Table 1.

	Baseline		48 h HCl diet	
Blood	WT	Cy/+	WT	Cy/+
pH	7.45 ± 0.01	7.44 ± 0.01	7.34 ± 0.01 ⁺⁺⁺	7.13 ± 0.00 ^{*****}
pCO ₂ , mmHg	36.9 ± 1.1	31.3 ± 0.3 ^{**}	30.1 ± 0.6 ⁺⁺	26.3 ± 1.2 ⁺⁺⁺
pO ₂ , mmHg	90.9 ± 1.4	93.4 ± 1.4	96.7 ± 1.1 ⁺⁺	95.3 ± 4.3
HCO ₃ ⁻ , mM	25.2 ± 0.7	20.6 ± 0.2 ^{***}	15.6 ± 0.3 ⁺⁺⁺	8.3 ± 0.4 ^{*****}
SO ₂ , %	95.4 ± 0.3	95.6 ± 0.2	95.2 ± 0.1	93.3 ± 0.8 [*]
Hematocrit	43.9 ± 2.9	40.6 ± 2.1 ^{**}	46.0 ± 0.6	37.3 ± 0.7 ^{***}
Sodium, mM	139.0 ± 1.0	137.0 ± 0.4	145 ± 2 ⁺	145 ± 4 ⁺
Potassium, mM	3.8 ± 0.1	4.7 ± 0.1 ^{**}	5.4 ± 0.2 ⁺⁺	5.4 ± 0.3
Chloride, mM	100.0 ± 1.0	103 ± 1.0 [*]	106 ± 1 ^{*****}	115 ± 2 ^{*****}
Phosphate, mg/dl	6.8 ± 0.2	7.9 ± 0.3 ^{**}	6.9 ± 0.1	8.3 ± 0.4 ^{**}
Creatinine, mg/dl	0.3 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	1.3 ± 0.1 ^{*****}
BUN, mg/dl	15 ± 1	43 ± 2 ^{***}	14 ± 1	48 ± 3 ^{***}
Albumin, g/dl	1.8 ± 0.1	1.8 ± 0.0	1.9 ± 0.0	1.7 ± 0.1 ^{**}
Glucose, mg/dl	247 ± 18	197 ± 3 [*]	220 ± 7	173 ± 5 ^{*****}

Table 2.

	Baseline		24 h HCl diet		48 h HCl diet	
Urine	WT	Cy/+	WT	Cy/+	WT	Cy/+
pH	6.61 ± 0.03	5.74 ± 0.06***	5.73 ± 0.03 ⁺⁺⁺	5.48 ± 0.02 ^{***,++}	5.37 ± 0.03 ⁺⁺⁺	5.29 ± 0.02 ^{*,+++}
24h urine/body weight (ml/g)	0.09 ± 0.00	0.09 ± 0.01	0.11 ± 0.02 ⁺⁺	0.10 ± 0.01	0.10 ± 0.01	0.12 ± 0.00
Creatinine clearance, ml/min	8.3 ± 0.9	2.5 ± 0.2	n/a	n/a	3.68 ± 0.22 ⁺⁺⁺	0.82 ± 0.08 ^{***,+++}
Na ⁺ , mmol/24h	1.06 ± 0.12	1.33 ± 0.05	2.64 ± 0.17 ⁺⁺⁺	1.22 ± 0.10 ^{***}	2.18 ± 0.06 ⁺⁺⁺	1.33 ± 0.14 ^{***}
K ⁺ , mmol/24h	4.30 ± 0.36	6.77 ± 0.15 ^{***}	6.57 ± 0.31 ⁺⁺⁺	5.99 ± 0.27 ⁺	6.92 ± 0.16 ⁺⁺⁺	4.50 ± 0.29 ^{***,+++}
Phosphate, mmol/24 h	0.63 ± 0.03	0.96 ± 0.04 ^{***}	1.05 ± 0.08 ⁺⁺⁺	1.08 ± 0.07	1.18 ± 0.05 ⁺⁺⁺	1.27 ± 0.07 ⁺⁺
NH ₄ ⁺ , mmol/24 h	0.11 ± 0.01	0.07 ± 0.00	3.18 ± 0.22 ⁺⁺⁺	1.10 ± 0.24 ^{***,++}	8.32 ± 0.25 ⁺⁺⁺	1.71 ± 0.09 ^{***,++}
HCO ₃ ⁻ , mmol/24 h	5.78 ± 0.44	1.17 ± 0.13 ^{***}	0.29 ± 0.04 ⁺	0.39 ± 0.03 ⁺⁺	0.18 ± 0.02 ⁺⁺⁺	0.22 ± 0.01 ⁺⁺⁺

FIGURE LEGENDS

Table 1

Summary of arterial blood parameters from wildtype and cy/+ CKD rats on standard diet or after 2 days of HCl-diet. Values are means \pm SE; n=5-6 animals/group, *,+ p < 0.05, **,++ p < 0.01, ***,+++ p < 0.001, * significantly different between wildtype and CKD rats (cy/+) at the same time point, + significantly different between baseline and 48h HCl diet for the same genotype.

Table 2

Summary of urine parameters from wildtype and cy/+ CKD rats on standard diet or after 2 days of HCl-diet. Values are means \pm SE; n=3-6 animals/group, *,+ p < 0.05, **,++ p < 0.01, ***,+++ p < 0.001, * significantly different between wildtype and CKD rats (cy/+) for the same time point, + significantly different between baseline and/or 24h HCl and/or 48h HCl diet for the same genotype.

Figure 1. Reduced mRNA expression of key ammoniagenic molecules in kidneys from CKD rats

Normalized mRNA abundance of PEPCK, SNAT3, PDG, and NHE3 in kidneys from controls and CKD rats (cy/+) under standard diet and 2 days of acid load with HCl. n=5-6 per group, * p < 0.05, ** p < 0.01, *** p < 0.001 significantly different between wildtype and CKD rats (cy/+).

Figure 2. Altered expression of key molecules of ammoniagenesis in kidneys from CKD rats

Protein abundance of PEPCK, SNAT3, PDG, and NHE3 are shown in controls and CKD rats (cy/+) on standard diet and after 2 days of acid load with HCl. The immunoblots were stripped and reprobed for all proteins and β -actin. All data were normalized against β -actin. n=5-6 per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different between wildtype and CKD rats (cy/+).

Figure 3. Altered expression of the proximal tubular bicarbonate transporter NBCe1

Normalized mRNA and protein abundance of NBCe1 are shown in controls and CKD rats (cy/+) on standard diet and after 2 days of acid load with HCl. Immunoblots were normalized against β -actin. n=5-6 per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different between wildtype and CKD rats (cy/+).

Figure 4. mRNA expression of sodium dependent phosphate transporters is altered in CKD rats

Normalized mRNA abundance of NaPi-IIa, NaPi-IIc, and Pit-2 are shown in controls and CKD rats (cy/+) on standard diet and after 2 days of acid load with HCl. n=5-6 per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different between wildtype and cy/+.

Figure 5. Expression of renal sodium dependent phosphate transporters in CKD rats

Protein abundance of NaPi-IIa, NaPi-IIc, and Pit-2 are shown in controls and CKD (cy/+) rats on standard diet and after 2 days of acid load with HCl. The immunoblots were stripped and reprobed for all proteins and β -actin. All data were normalized against β -actin. n=5-6 per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different between wildtype and CKD rats (cy/+).

Figure 6. Regulation of mRNA abundance of collecting duct acid-base transporters

Normalized mRNA abundance of AE1, pendrin, RhCG, and B1 subunit of the H^+ -ATPase are shown in controls and CKD rats (cy/+) on standard diet and after 2 days of acid load with HCl. n=5-6 per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different between wildtype and CKD rats (cy/+).

Figure 7. Reduced expression of the ammonia transporters RhCG

Protein abundance of AE1, pendrin, RhCG, and the B1 subunit of the H^+ -ATPase are shown in controls and CKD rats (cy/+) on standard diet and after 2 days of acid load with HCl. The immunoblots were stripped and reprobed for all proteins and β -actin. All data were normalized against β -actin. n=5-6 per group, * $p <$

0.05, ** $p < 0.01$, *** $p < 0.001$ significantly different between wildtype and CKD rats (cy/+).

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Figure 1

Impaired ammonium excretion in CKD

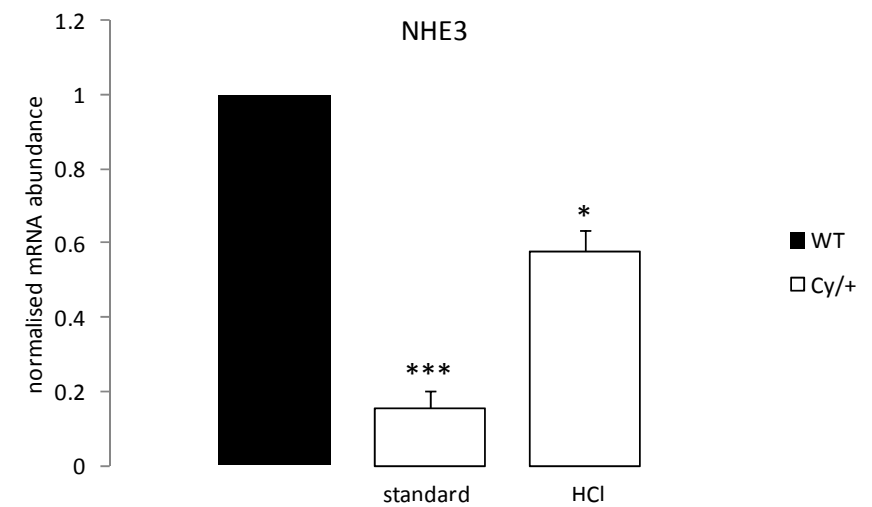
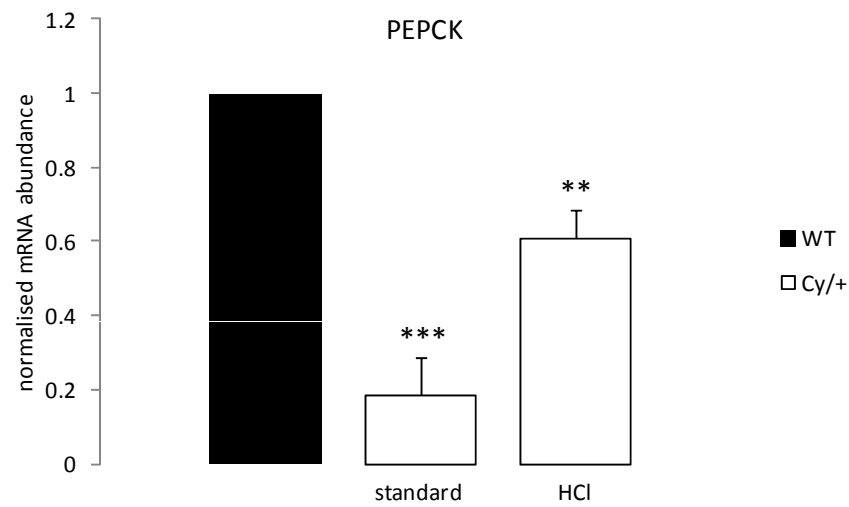
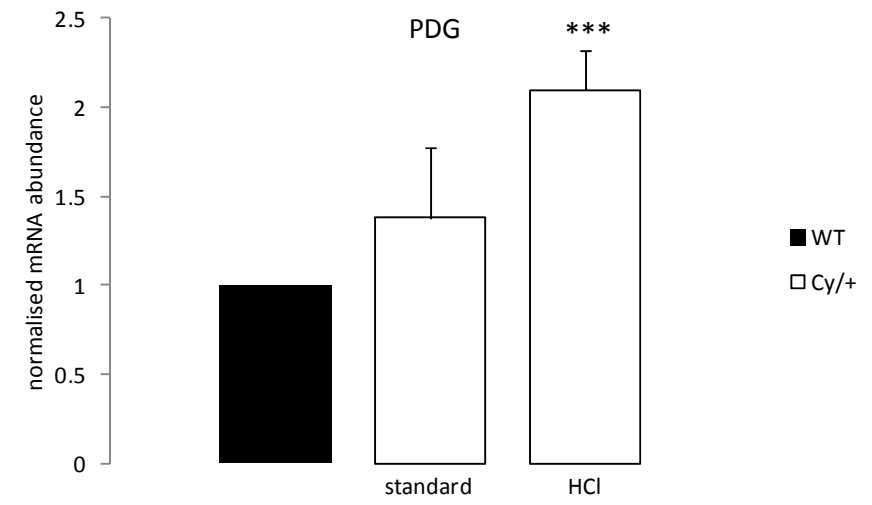
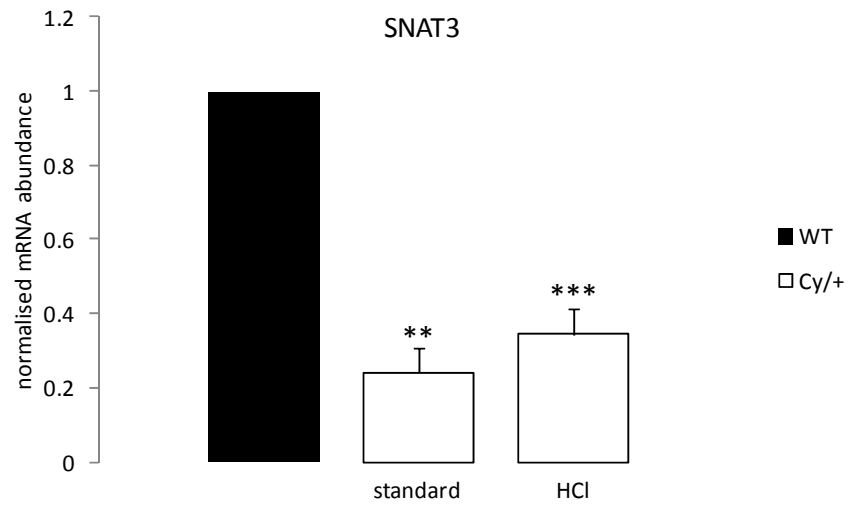


Figure 2

Impaired ammonium excretion in CKD

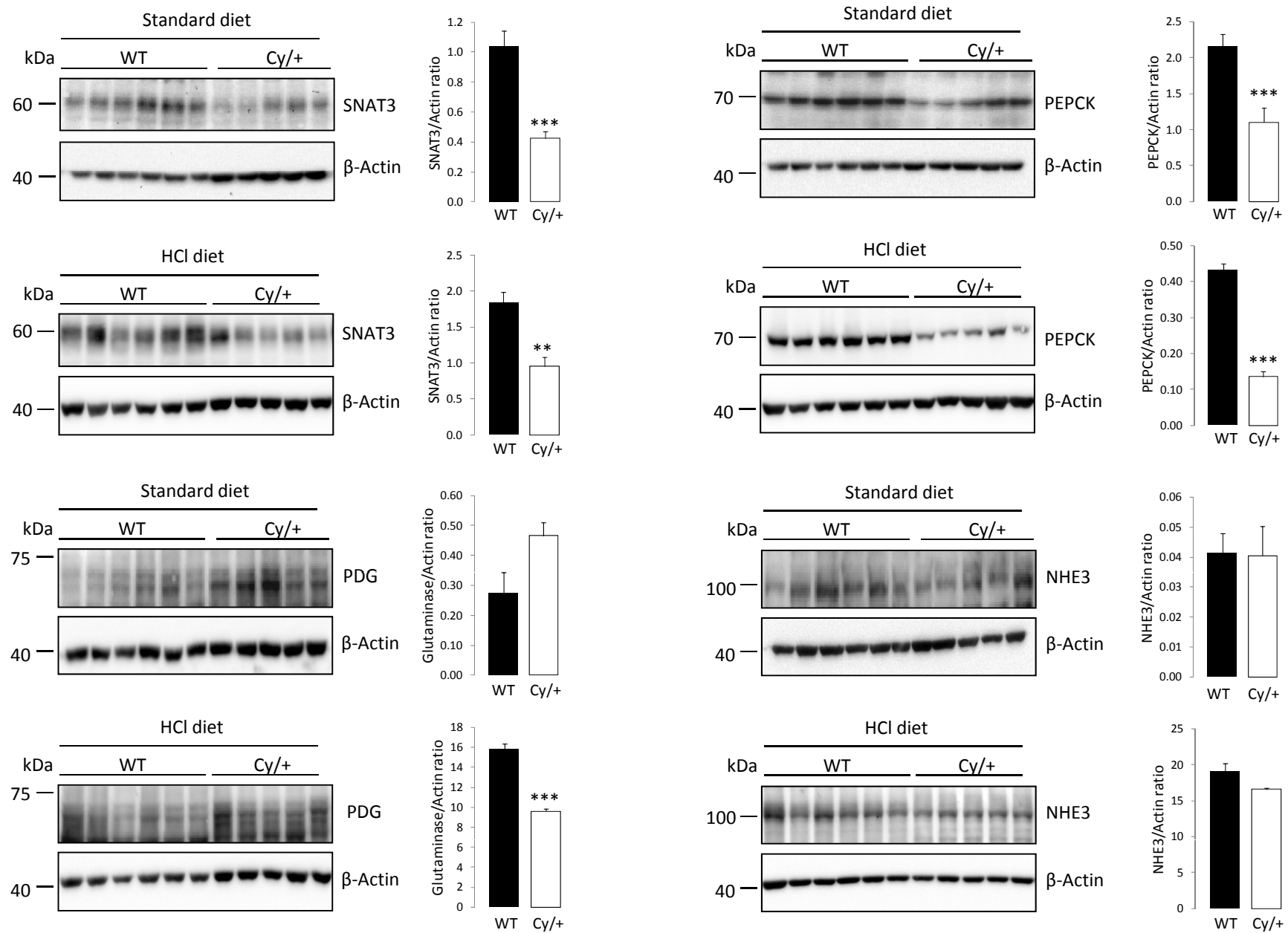


Figure 3

Impaired ammonium excretion in CKD

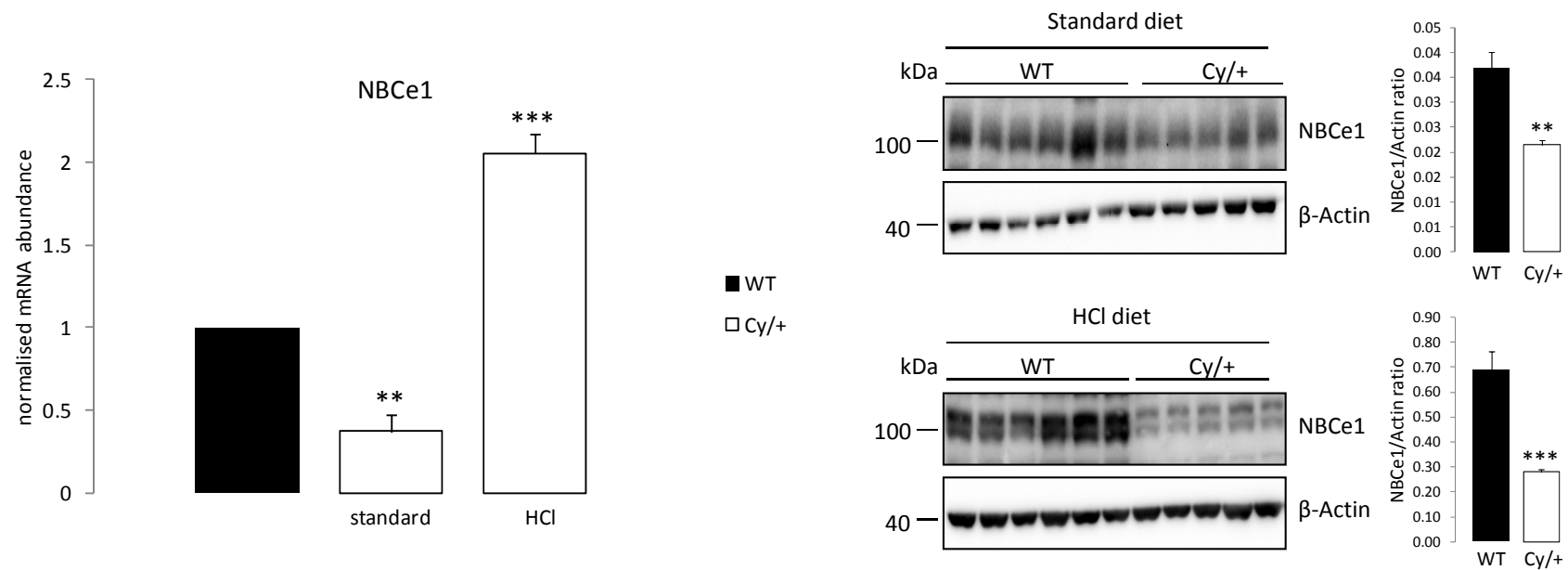


Figure 4

Impaired ammonium excretion in CKD

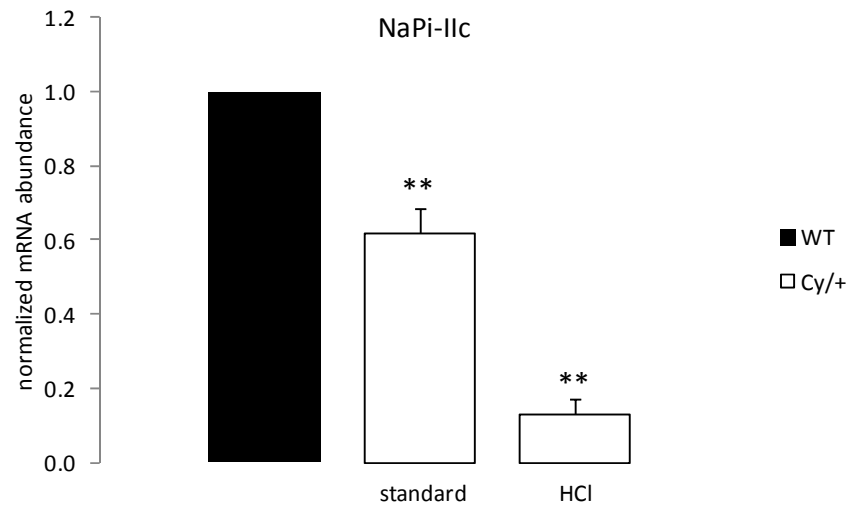
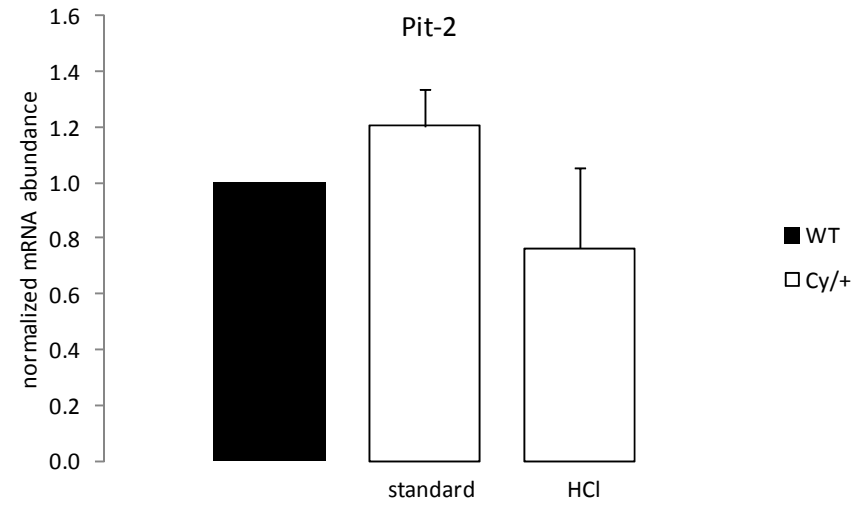
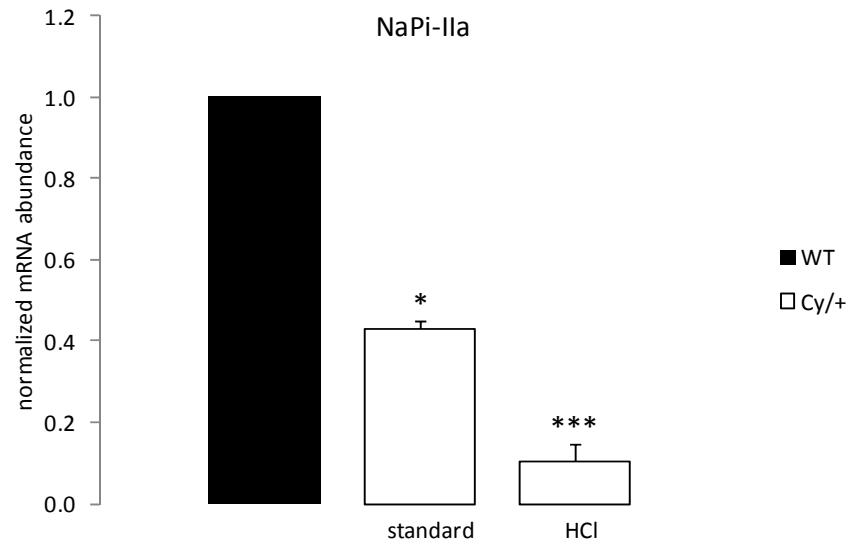


Figure 5

Impaired ammonium excretion in CKD

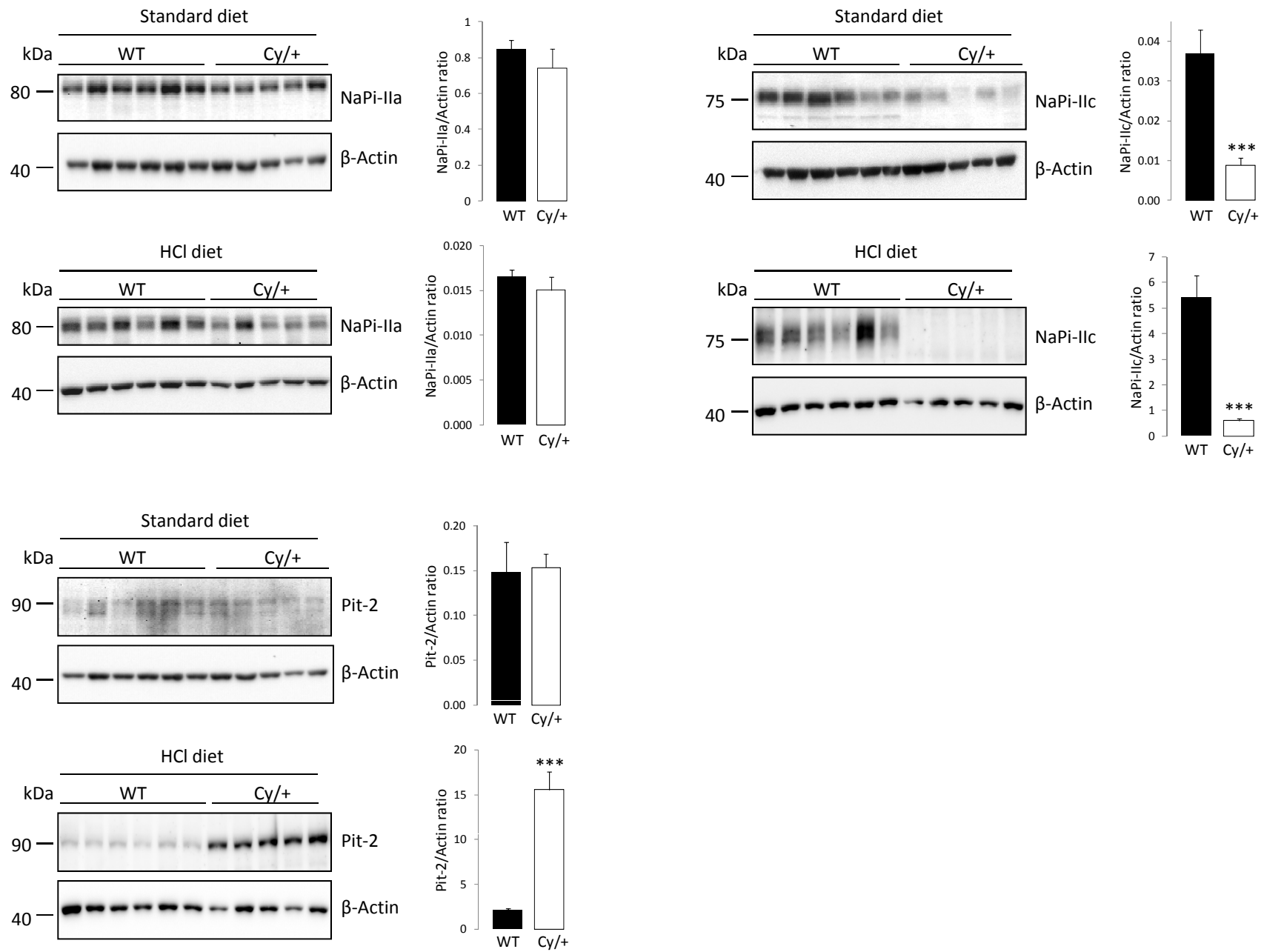


Figure 6

Impaired ammonium excretion in CKD

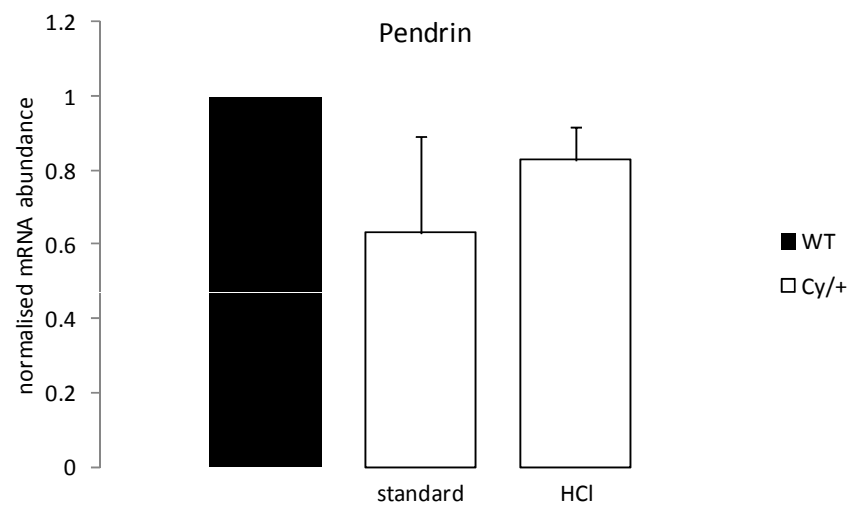
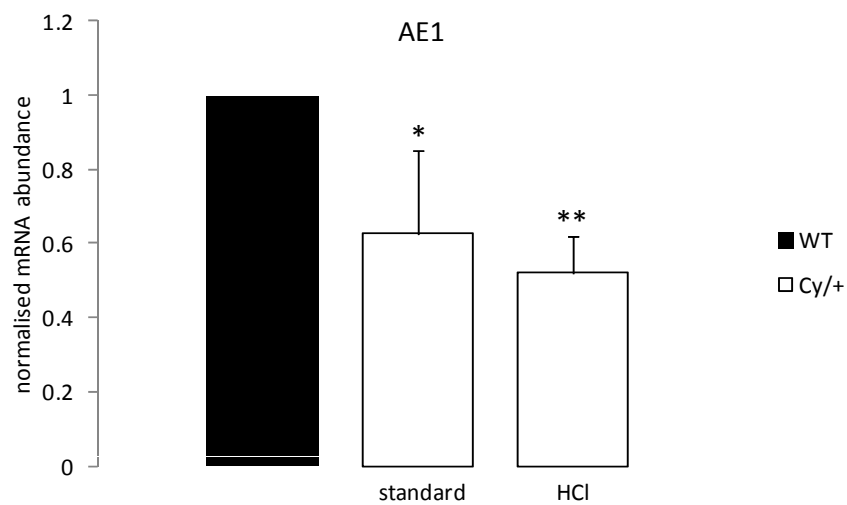
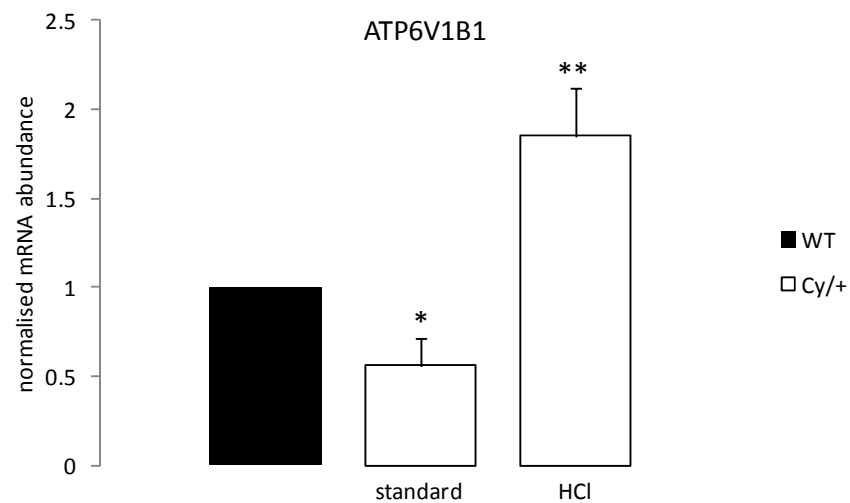
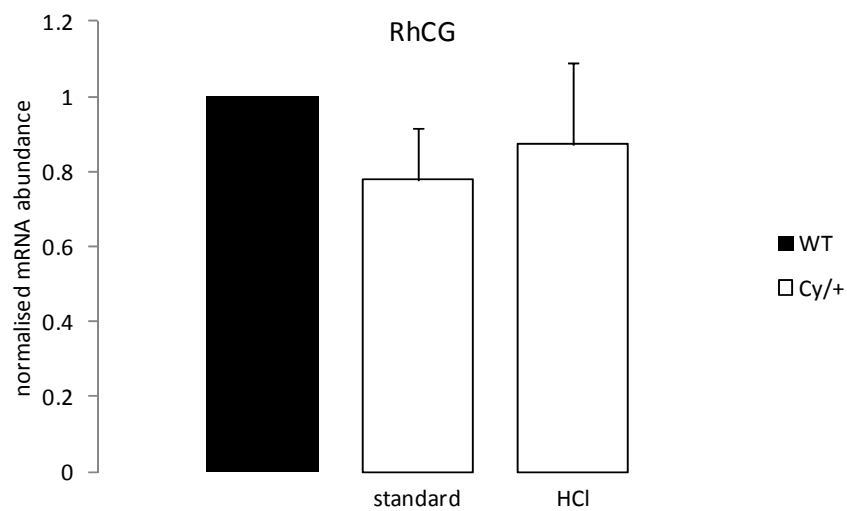
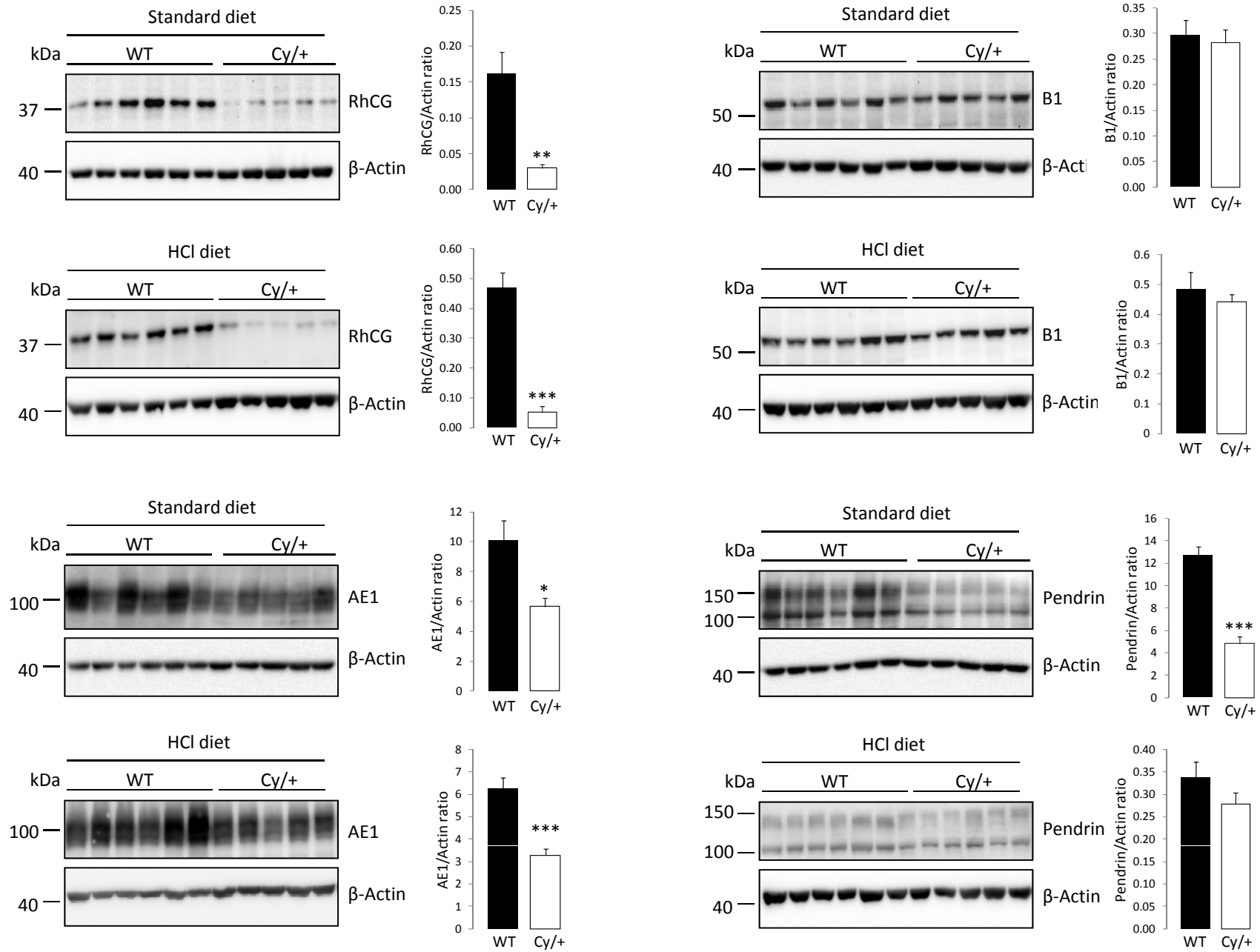


Figure 7

Impaired ammonium excretion in CKD



Supplementary table 1. List of primary antibodies

1° Antibody	Host	Dilution	Source (Ref)
β -Actin	mouse	1:20000	(1)
AE1	rabbit 1, day 115	1:10000	(2)
Atp6v1b1 (B1)	rabbit 1, day 90	1:5000	(3)
PDG (Glutaminase)	rabbit	1:5000	(4)
NaPi-IIa	rabbit	1:6000	(5)
NaPi-IIc	rabbit	1:3000	(6)
NBCe1	rabbit SG-3341	1:10000	(7)
NHE3	rabbit 2, day 115	1:5000	(8)
Pendrin	rabbit 1, day 115	1:5000	(9)
PEPCK-C	rabbit	1:5000	(10)
Pit-2	rabbit	1:3000	(11)
RhCG	mouse	1:5000	(12)
SNAT3	rabbit	~1:667	(13)

Supplementary table 2. Primers and probes used for semi-quantitative real-time RT-PCR

Gene	Acc. No.	Primers	Probe
Slc4a1 (AE1)	NM_012651.2	Fw: 5'-GCC ATG ATG CTA CGA AAG TTC A-3' Rev: 5'-GGA CCA TTA TCA GGA TGG AGA TG-3'	5'-CCT ACT TCC CCG GCA AGC TAC GCA G-3'
Atp6v1b1 (B1)	NM_001107867.2	Fw: 5'-GTC AAT GGG CCT CTG GTG-3' Rev: 5'-CCC TCA AAC ACC TGA ACA ATG-3'	5'-CTC AGA GGA GTG GCC AAG TGC TTG A-3'
PDG (Glutaminase)	NM_012569.2	Fw: 5'-CTT CTC AGG GCA GTT TGC ATT-3' Rev: 5'-TTC CTT TAA CAC TGT TGC CCA TCT-3'	5'-CTT CCT GCA AAA TCT GGA GTT GCT GGG-3'
Slc34a1 (NaPi-IIa)	NM_013030.1	Fw: 5'-GGA ATC ACA GTC TCA TTC GGA TT-3' Rev: 5'-ATG GCC TCT ACC CTG GAC ATA G-3'	5'-TGT CAA CCA GAG ACA AAA GAG GCT TCC ACT-3'
Slc34a3 (NaPi-IIc)	NM_139338.1	Fw: 5'-GGG ATC GGG ATG AAT TTC AGA-3' Rev: 5'-GGG CCA GCT CAC TCA GTC TCT-3'	5'-ACG GCA TCT TCA ACT GGC TCA CAG TGT T-3'
Slc4a4A+B (NBCe1)	NM_053424.1	Fw: 5'-GGT GTG GAT ACT CCG AAG CTA ATT-3' Rev: 5'-CAA AGG GTG GGA CAA ACC AA-3'	5'-AGT TCA AGC CAA CAA GCC CTC ACC G-3'
Slc9a3 (NHE3)	NM_012654.1	Fw: 5'-CAC CCA CCA CAC GTT GCA-3' Rev: 5'-GTG AGC TCG TGC CGA CTG T-3'	5'-CTC TAC AAG CCT CGG CAG GAG TAC AA-3'
Slc26a4 (Pendrin)	NM_019214.1	Fw: 5'-CGC CAC CAA GCA TGA CTA CA-3' Rev: 5'-TCC TGG ACA GCC GTT CGA-3'	5'-AAC CAG GAA TTC ATT GCC TTC GGG ATA A-3'
Pck1 (PEPCK-C)	NM_198780.3	Fw: 5'-AGT CAC CAT CAC TTC CTG GAA GA-3' Rev: 5'-GGT GCA GAA TCG CGA GTT G-3'	5'-AGT GGA GAC CAC AGG ATG AGG AAC CGT-3'
Slc20a2 (Pit-2)	NM_017223.2	Fw: 5'-CCT GCT TTG GGT CCT TTG C-3' Rev: 5'-CAG CCA CAG GGC CAC AAG-3'	5'-ACG GTG GCA ACG ATG TGA GCA ATG-3'
Rhcg (RhCG)	NM_183053.1	Fw: 5'-CTC CTT GGC AGC TAG TGT GCT A-3' Rev: 5'-CCT GCA AGT GTG GCG TTC T-3'	5'-AAG AAG GGC AAG TTG GAT ATG GTG CAC A-3'
Slc38a3 (SNAT3)	NM_145776.1	Fw: 5'-CCT GAG GTG CTG CCC ATA TAT AC-3' Rev: 5'-GCA ATG GAC AGG TTG GAG ATG-3'	5'-TCA AGG ACC CCT CCA AGA GGA AGA TGC-3'
18S rRNA	4310893E	Applied Biosystems	

References for antibodies

- (1) Sigma-Aldrich (Monoclonal Anti- β -Actin, antibody produced in mouse, clone AC-74, ascites fluid, Product No. A5316)
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